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LC ESI-MS and FT-IR Analysis of *Dendrophthoe pentandra* L. Miq Leaf Methanolic Extracts to Identify Compounds with Progesterone-Like Effects

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Abstract: An earlier study found that progesterone hormone levels in adult female rats increased by nearly two-fold relative to usual levels following intramuscular injection (w/v, 100 mg/kg body weight for four days (s.d.d.)) of methanol extracts of *Dendrophthoe petandra* L. Miq (common name *Benalu duku*) leaves. This result suggests that *Benalu duku* contains pregnane derivative steroids that have carbon bonding at positions 1 and 21. Here we examined the specificity and pharmacodynamics of active substances with progesterone-like effects in crude methanol extracts made from *Benalu duku* leaves. Pulverized *Benalu duku* leaves (400 g) were extracted with 2 L analytical grade methanol at 20°C for 72 h using a shaker maceration method. The semi-solid crude extract was dried under vacuum and exposed to UV light prior to spectroscopic analysis. FT-IR and LC ESI MS analysis detected active substances having a pregnane derivative chemical structure with progesterone, medroxy progesterone acetate, megestrol acetate and dydrogesterone present at distributions of about 30, 66, 3 and 1%.

Key words: *Benalu duku*, pregnane derivate, anabolic steroid, leaf extract FT-IR, Methanolic leaf extract LC-ESI MS

INTRODUCTION

Good nutrition requires a matrix of essential nutrients that are necessary to maintain human health. Nutritional imbalances, as well as disruptions in hormone levels, can cause several diseases that range from benign to malignant cancer. Herbal medicines have been predicted to have potential applications to treat human cancers. Indeed, some studies showed that plants produce beneficially active substances such as alkaloids, flavonoids, polyphenols, terpenoids and free steroid (Arung *et al*., 2009; Ang *et al*., 2014; Arun *et al*., 2014).

The parasitic plant that grows on *Lancium demesticum*, *Dendrophthoe pentandra* L. Miq (*Benalu duku*), is known to have several essential active substances that can affect cell proliferation (Kwanda *et al*., 2013; Lazuardi and Bambang, 2014). A previous report showed that crude methanolic extracts of *Benalu duku* leaves administered to adult female rats can increase progesterone hormone levels by more than two-fold relative to usual levels, while FSH levels were unaffected (Lazuardi and Bambang, 2014). Furthermore, other studies found that *Benalu duku* contains several substances that have progesterone-like effects (Ogbuewu *et al*., 2011; Cooper and Page, 2014).

Progesterone-like effects are thought to be associated with the specific chemical structure of a pregnane derivative that includes double-bonded carbons at positions 1 to 21, or (8S,9S,10S,13R,14S,17S)-17-ethyl-10,13-dimethyl-2,3,4,5,6,7,8,9,11,12,14,15,16,17-tetradecahydro-1H-cyclopenta [a] phenanthrene (Sivils *et al*., 2011; Del Pup *et al*., 2014). Pregnane derivatives share specific anabolic steroid structures that are found in cortisone, hydrocortisone, progesterone, medroxy progesterone acetate, megestrol acetate, 17-α- hydroxyl progesterone acetate and dydrogesterone. Several of these anabolic hormones have had therapeutic applications in prostate cancer (Cooper and Page, 2014).

The subtropical and tropical plant *Avicennia germinans* is similar to *Benalu duku* in that methanol extracts of both plants are thought to contain anabolic steroids. (Mori *et al*., 2015). Here we used LC-ESI MS and FT-IR spectroscopy to examine whether *Benalu duku* leaf extracts contain progesterone-like molecules such as progesterone, medroxy progesterone acetate, megestrol acetate and dydrogesterone (Kind and Fiehn *et al*., 2011).

MATERIALS AND METHODS

Herbal medicine and extraction method: *Benalu duku* leaves were obtained from the Muara Enim district of the West Sumatra province of Indonesia. The Botany Research Institute in Tangerang-Jakarta Indonesia

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confirmed that the leaves originated from *Dendrophthoe petandra* L. Miq (Lazuardi and Bambang, 2014). Reference progesterone and megestrol acetate were obtained from Sigma-Aldrich (St. Louis, MO USA, Product No. 46665, Batch SEBA XV and Product No. 46420, Batch SZB9173XV, respectively). Pharmaceutical grade reference medroxy progesterone acetate was obtained from Harsen Pharmaceutical Industry (Jakarta, Indonesia). Reference dydrogesterone was from the European Directorate for Quality of Medicines and Healthcare (Strasbourg, France) under Catalog Code Y0001004.

Benalu duku leaves (400 g) were pulverized and 2 L analytical grade methanol was added. The mixture was divided into two 1 L flasks and extracted for 72 h at 20°C using the movement shaker maceration method. The semi-solid crude extract was completely dried under vacuum and exposed to UV light for 15 min to eliminate fungal and bacterial contaminants.

FT-IR and LC ESI MS spectroscopy: To determine specific molecular structures of compounds present in *Benalu duku* leaves, samples of semi-solid crude extracts were subjected to FT-IR using a Perkin-Elmer Frontier 89485 spectrometer equipped with a MIR TGS detector operating at an Optical Path Distance (OPD) velocity of 0.20 cm/sec. To examine molecular characteristics of progesterone-like substances, spectra were acquired between 400 to 4000/cm (Fackler *et al*., 2010, Jianhua *et al*., 2012).

LC-ESI MS spectra were obtained using an Accela TSQ Quantum Access apparatus (Thermo Scientific) operating with the following specifications: Column: Hypersil GOLD, 0.2 µm particle size, length 10 cm, Gradient: mobile phase solution A 0.1% in Aqua pro chromatograph and B 0.1% formic acid in acetonitrile pro HPLC with solution B increasing from 35 to 70% in 20 min; UV detection: 254 nm. Automatic uptake and injection capacity were adjusted to 10 µl; the flushing capacity was 400 µl with 100 µl/sec velocity; velocity injector speed was set to 8 µl/sec; the suction apparatus had a 2 ml capacity and a 1.2 auto sample vial was used. The column temperature was maintained at 22°C and the maximum pump pressure was 1250 PSI with an assay pressure of 10 BAR (Kushnir *et al*., 2008; Ono *et al*., 2008; Kind and Fiehn, 2010; Batchu *et al*., 2013).

RESULTS

FT-IR analysis of crude methanol *Benalu duku* leaf extracts showed aromatic compound stretching with specific overtones ranging from 1650 to 2000/cm (Table 1 and Fig. 1) The 1550/cm to 1600/cm region also showed aromatic stretching (1 s). Meanwhile, s orbital stretching was seen between 1450 and 1500/cm. These spectral features are consistent with the presence of aromatic molecules. In the fingerprint area, wavelength numbers of 600 to 900/cm and 1000 to 1300/cm

Table 1: Infra-red spectra (cm^{-1}) and intensity (%T) of crude *Benalu duku* methanol extract and reference materials with progesterone like-effects

	------ CRM Dydrogesterone ------		---- CRM Megestrol acetate ----		CRM Med. Prog Acet.		-------- CRM Progesterone --------		-- *Benalu duku* extract --	
Identified function group	T (%)	cm^{-1}	T (%)	cm^{-1}	T (%)	cm^{-1}	T (%)	cm^{-1}	T (±%)	cm^{-1}
O-H	14.17-14.58	3435.72-3374.34	10.16	3436.39	0.11	3434.34	72	3436.69	15-11	3500-3400
Asymmetrical vibration, stretching C-H	5.05-13.35	2989.16-2930.19	12.04-12.17	2946.49-2927.66	-	Absent	10.25-8.22	2969.05-2925	12.87	2927.33
Stretching aromatic ring (1,s)	1.24-2.94	1659.51-1620.82	6.49-10.9	1664.2-1629.09	1.05	1638.95	49-8.83	1699.13-1616.17	17.15	1632.88
Stretching aromatic ring (s)	14.48	1452.06	14.88	1458.7	3.29	1401.19	15.45	1438.76	18.49	1458.07
Flexible C-H on orbital (l)	12.4	1277.7	10.1	1269.83	-	Absent	17.85-18.67	1279.25-1268.89	19.21	1272.22
Alkene, R-OH, stretching vibration C-H, aromatic	7.97	1193.1	14.18	1206.31	-	Absent	14.97	1204.91	19.36	1203.09
-CH-CH-(trans), R-CH2	17.08-14.51	1174.79-1162.58	16.27	1166.41	-	Absent	17.64-16.36	1179.06-1162.48	19.73	1169.44
C = CH2, mono substitute alkenes	21.79	1064.16	16.29-15.81	1083.18-1058.62	3.28	1112.60	19.96	1116.32	19.17	1062.81
Tri substitute alkenes, meta-di substitute Benzene (aromatic)	24.45-23.51	831.21-793.86	14.9-21.26	877.33-796.78	-	Absent	17.15	871.18	23.84	826.55
Cis-disubstitute alkenes, mono substitute benzene, meta-disubstitute benzene (Aromatic)	23.21	727.77	20.62	755.83-713.19	-	Absent	21.77-21.17	778.14-687.31	23.67	721.48
Cis-disubstituted alkenes (vinyl, C-H)	18.55	630.99	20.65	634.94	2.57	644.79	21.17	687.31	23.8	639.21

Table 2: ESI retention time and ionized molecules ion of ESI of crude methanol *Benalu duku* leaf extracts and medroxy progesterone acetate, progesterone, megestrol acetate and dydrogesterone reference molecules

Analytes	Weight (µg/ml)	Area	Retention time (min)	Electron spray Ionization (ESI) (m/z)
Crude *Benalu duku* leaf extract	0.0646	227044	4.40	387.000
Medroxy progesterone acetate	0.15	99585	4.40	387.000
Crude *Benalu duku* leaf extract	0.06425	9472	4.46	315.000
Progesterone	0.075	25385	4.46	315.000
Crude *Benalu duku* leaf extract	0.0646	16034	4.38	385.000
Megestrol acetate	0.069	319900	4.39	385.000
Crude *Benalu duku* leaf extract	0.0646	49216	4.30	313.000
Dydrogesterone	0.0138	725646	4.31	313.000

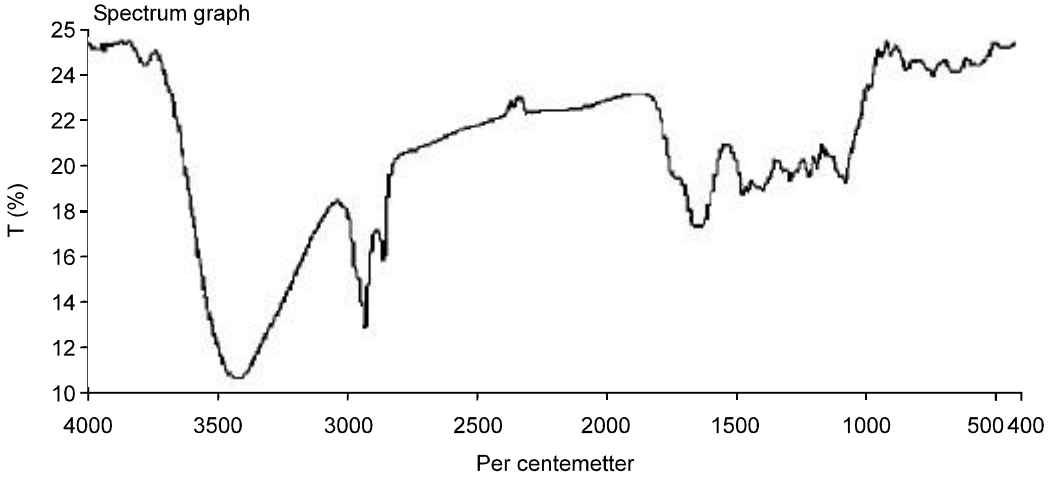

Fig. 1: IR Spectrum of crude *Benalu duku* leaf methanol extract

indicated the presence of flexible carbon atoms and hydrogen outside area (k orbital) and inside area (l orbital). The IR spectra of the reference molecules and *Benalu duku* leaf extracts at high intensity (%T) indicating a wavelength number of 1400 to 1050/cm and 1600 to 1660/cm were largely identical. Even at low intensity (%T), the fingerprint area between 750 and 1000/cm of the reference and extract samples was identical.

LC ESI-MS analysis on a Triple Stage Quadruple mass spectrometer showed progesterone-like compounds in *Benalu duku* leaf extracts that were identical to medroxy progesterone acetate, progesterone, megestrol acetate and dydrogesterone reference samples. The dependent variables retention time and ion molecules in electrospray ionization (ESI, m/z) analysis of *Benalu duku* leaf extracts were also similar to that of the reference molecules (Table 2). Moreover, LC ESI-MS spectra obtained from *Benalu duku* leaf extracts showed the presence of substances identical to medroxy progesterone acetate, progesterone, megestrol acetate and dydrogesterone (Fig. 2-5). Thus, the LC ESI-MS results indicate that active substances present in *Benalu duku* could have pharmacodynamic actions that stimulate release of progesterone-like hormones (Lazuardi and Bambang, 2014).

DISCUSSION

FT-IR analysis of methanol extracts prepared from leaves of the parasitic plant *Benalu duku* showed the presence of several functional groups in the 1200 to 1600/cm region of the spectrum (Table 1, Fig. 1). These functional groups could be assigned as follows: N-H bend or C = C stretch, amine or alkane; C-N bend, amides; C-N stretch and C-O stretch and C = S stretch, aromatic amines and carboxylic acids, respectively the characterization of specific functional molecules in the fingerprint area for *Dendrophthoe* species such as *Benalu duku* or other parasitic plants by FT-IR analysis showed similar results (Table 1) (Fackler *et al*., 2010; Jianhua *et al*., 2012; Ameer *et al*., 2015).

Analysis of *Benalu duku* leaf extracts in terms of LC ESI-MS retention time and ESI (m/z) indicated the presence of two progesterone-like substances that were identical to reference spectra for medroxy progesterone acetate and progesterone. The spectra were consistent with the presence of the progesterone-like substances megestrol acetate and dydrogesterone in the extracts, although the retention time shifted by 0.01 and 0.6 minutes, respectively, relative to the reference molecules. This shift could be due to polarity effects upon binding to particles in the Hypersil GOLD LC

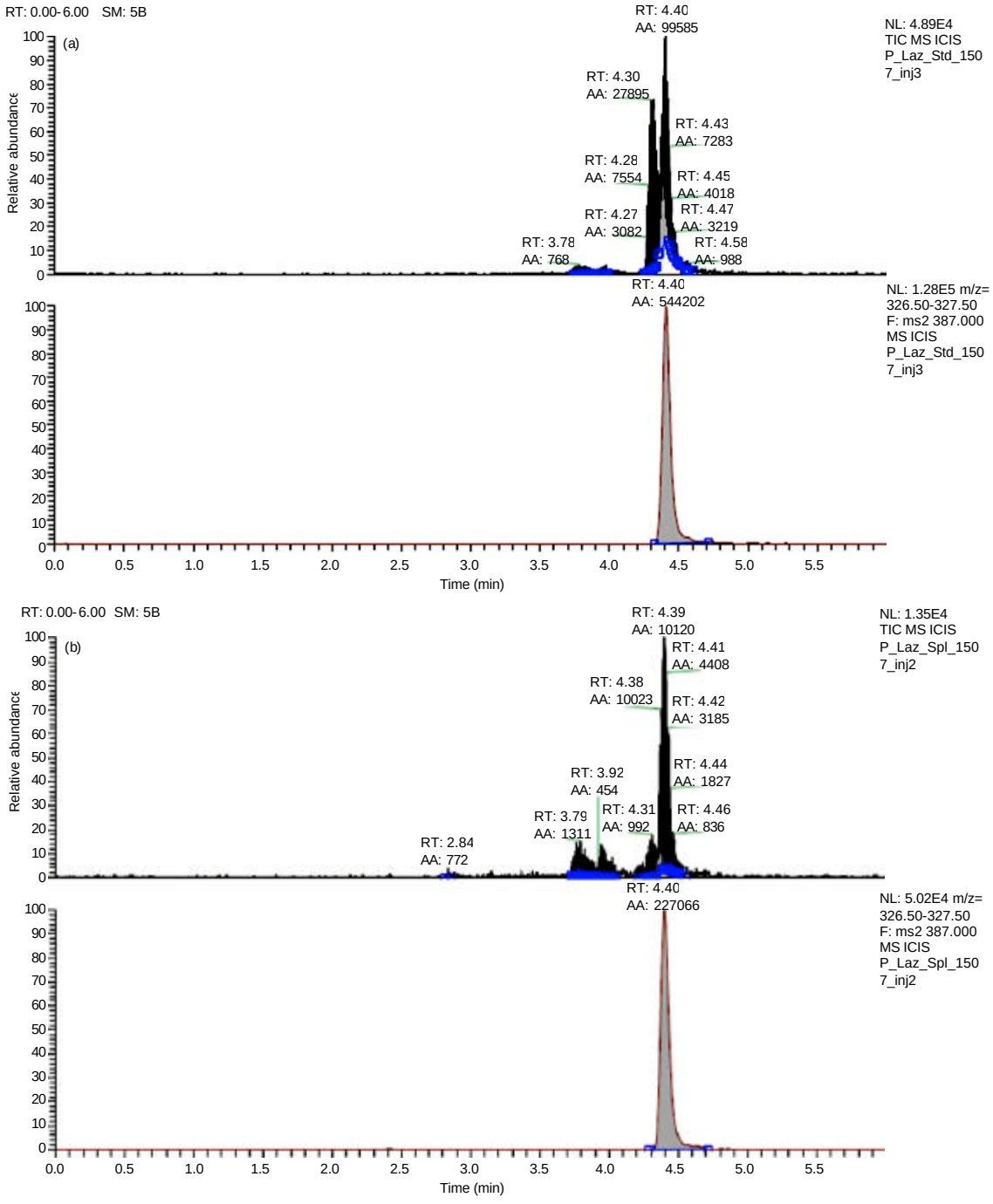

Fig. 2: LC ESI-MS 387.000 (m/z) spectra of a 0.150 µg/ml medroxy progesterone acetate reference solution (A) and Medroxy progesterone acetate (0.0646 µg/ml) identified in a crude extract of *Benalu duku* leaves (B)

column (Tuli and Ressom, 2009) and thus megestrol acetate and dydrogesterone in the extract would be identical to reference compounds. Overall, a comparison of the m/z and ESI (m/z) values for the reference materials and the progesterone-like substances in the extracts showed little difference ($p<0.05$), although the molecular ion fragment values were apparently lower than the ESI values (Table 3).

The differences in the values between the ESI and LC methods may be due to different sensing of ion molecule analytes after ionization of molecular ion fragments in the ionization chamber that occurs in the

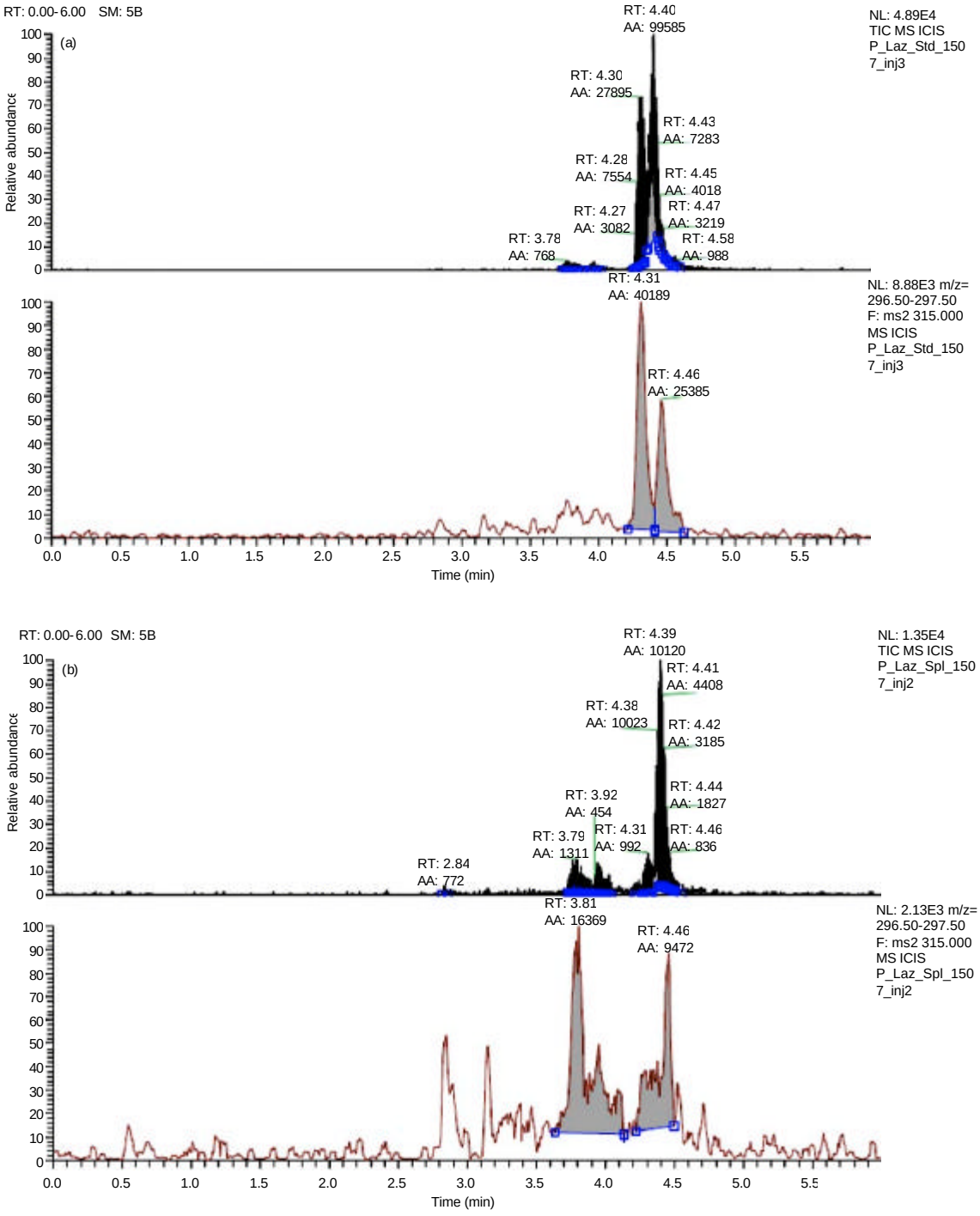

Fig. 3: LC ESI-MS at 315.000 (m/z) of reference progesterone (A) and progesterone present in a methanol extract of *Benalu duku* leaves (B)

ESI method. The ESI LC-MS Accela TSQ sensitivity in the mass range examined here is known to be m/z 3000 with Quantitation-Enhanced Data-Dependent MS/MS (QED-MS/MA), which is capable of monitoring up to three data points. Background signals can also be suppressed by correcting for the curved space after the ionization chamber (collision unit cell) that made 900. Meanwhile, the relative standard deviation (rsd) of the TSQ Quantum robustness of accession reached 2.9% (Zhang *et al*., 2008; Tuli and Ressom, 2009; Xingnan and Franke, 2011).

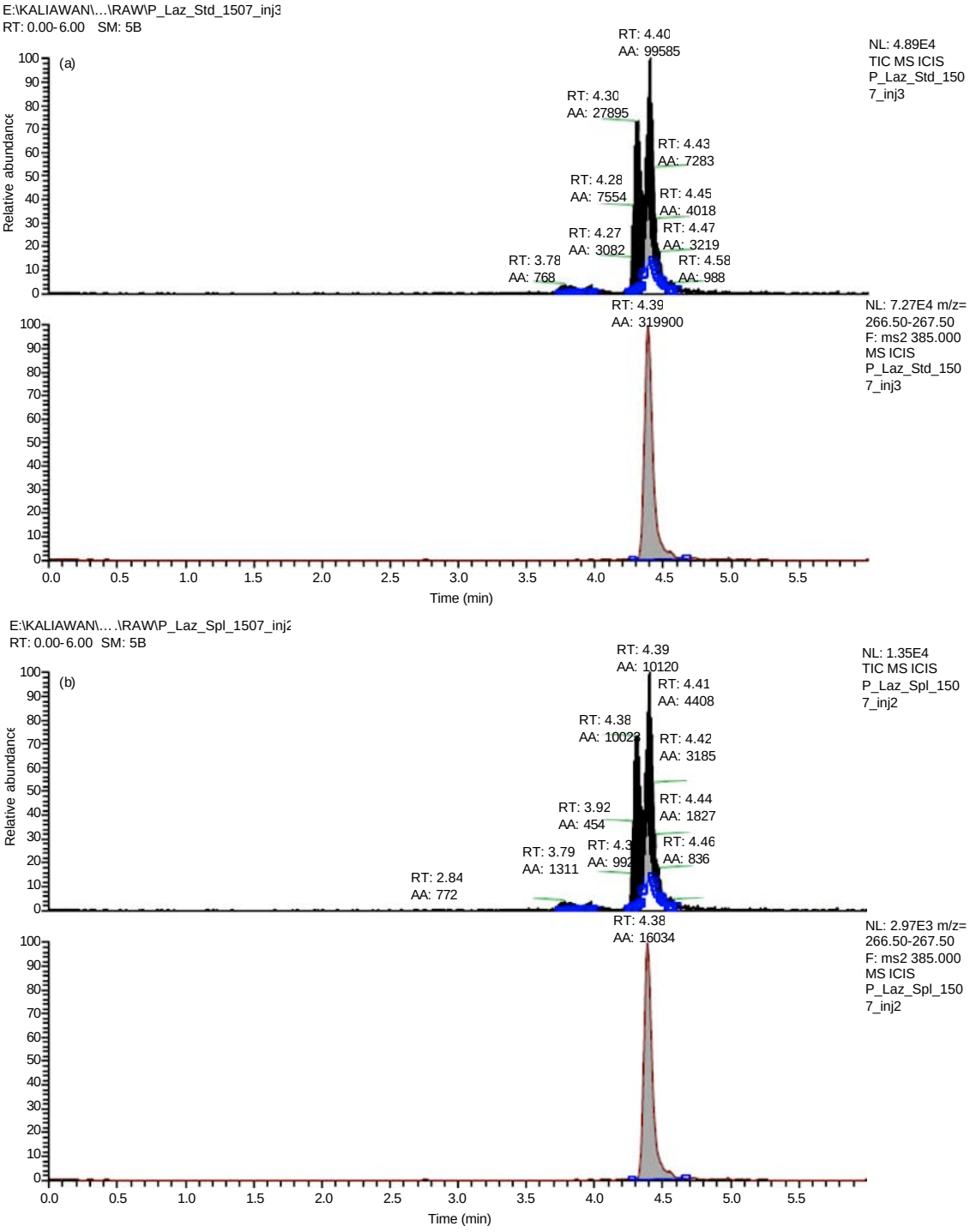

Fig. 4: LC ESI-MS 385.000 (m/z) of reference megestrol acetate (0.069 µg/ml) (A) and megestrol acetate (64.625 ng/ml) in the mobile phase eluent of a crude methanol extract of benalu duku leaves (B)

The spectra suggest that *Benalu duku* leaf extracts contain ~0.0656µg/ml progesterone-like substances, with medroxy progesterone acetate (0.062µg/ml) being the most common molecule, followed by progesterone (0.028µg/ml), megestrol acetate (0.003µg/ml) and dydrogesterone (0.0009µg/ml). This finding is consistent with our earlier study that showed the ability of *Benalu duku* to increase progesterone levels in adult female

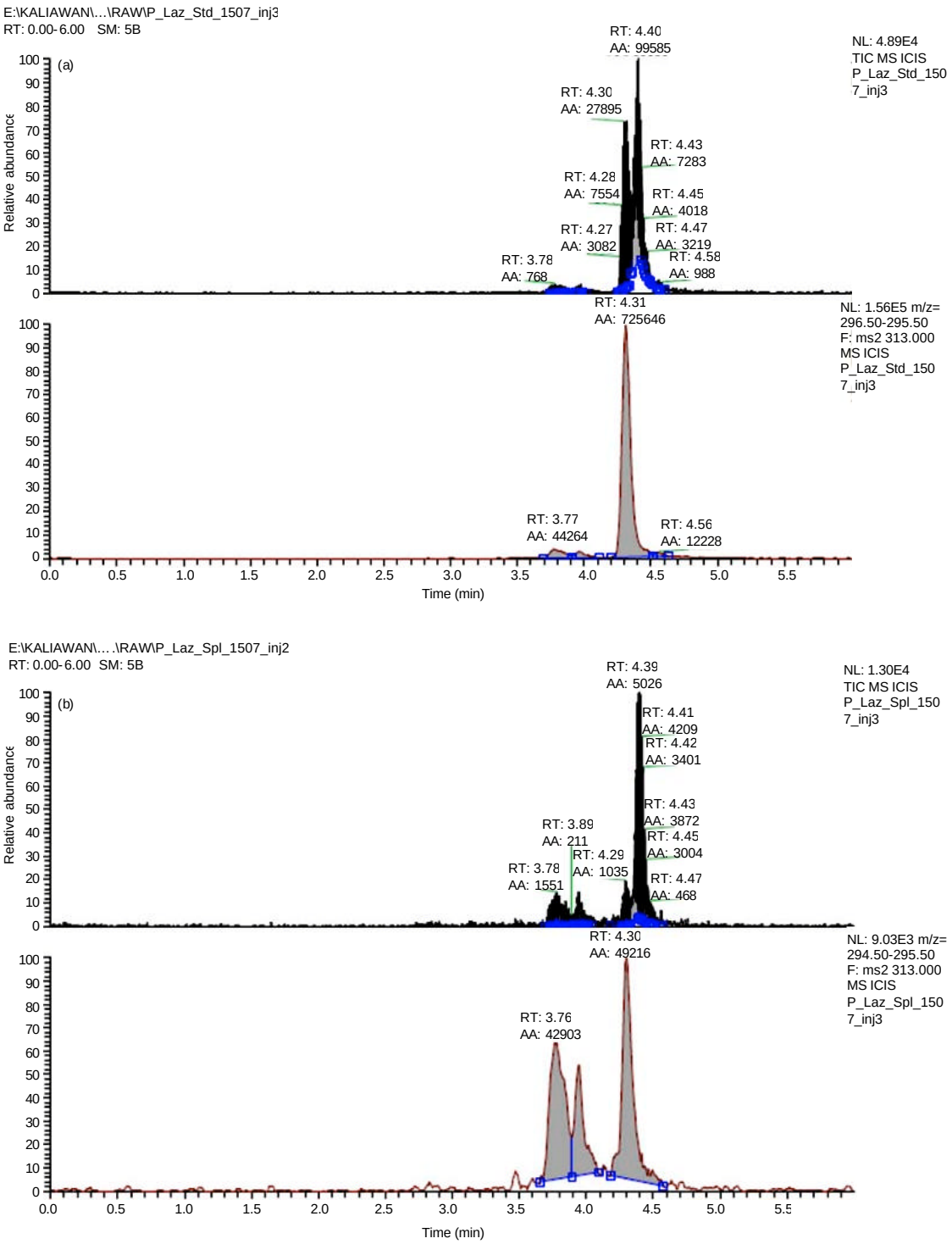

Fig. 5: LC ESI-MS 313.000 (m/z) of reference dydrogesterone (0.0138 µg/ml) (A) and dydrogesterone (0.0646 µg/ml) present in the mobile phase eluent of a crude methanol extract of *benalu duku* leaves (B)

rats by 2-fold (Lazuardi and Bambang, 2014). Similarly, another member of the *Dendrophthoe* species, *Chrysophyllum albidum*, was shown to contain bioactive compounds that negatively affected fertility (Sivils *et al*., 2011; Mori *et al*., 2015). Together these results suggest that a majority of parasitic plants in the *Dendrophthoe* species could contain androgenic steroids that have anti-fertility and progesterone-like effects.

Table 3: Molecular ion fragment and ESI values from samples and Certified Material Reference (CRM)

	Progesterone		Medroxy prog. Acet.		Meges. acetate		Dydrogesterone	
Parameters	CRM	Sample	CRM	Sample	CRM	Sample	CRM	Sample
Retention times (minutes)	4.46	4.46	4.4	4.4	4.39	4.38	4.31	4.30
Mol ion fragment (m/z)	296.50-297.50	296.50-297.50	326.50-327.50	326.50-327.50	266.50-267.50	266.50-267.50	294.50-295.50	294.5-295.5
ESI	315	315	387	387	385	385	313	313

Conclusions: Crude methanol extracts of *Benalu duku* leaves contained various progesterone-like substances with medroxy progesterone acetate, progesterone, megestrol acetate and dydrogesterone representing 66, 30, 3 and 1%, respectively, of the total. The absolute amount of identical compounds was obtained from medroxy progesterone acetate and progesterone. However, lower amounts of absolute identical compounds were obtained from megestrol acetate and dydrogesterone. Future studies should focus on isolating and identifying which active compound (s) with progesterone-like properties affects the physiology and hormone levels of animal subjects.

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